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14. ABSTRACT

Breast cancer is a highly diverse group of cancers and consists of at least 5 different subgroups. Furthermore, patients with Intra-tumor heterogeneity due to the presence of cancer cells with variable phenotypes such as different degrees of basal-like and luminal features increase the complexity of treatment. The aim of this project is to better understand the mechanisms that regulate breast cancer cell plasticity and origins of breast cancer heterogeneity. Our focus is the function of protein kinase D1 (PKD1), which is a serine/threonine kinase. We previously showed that PKD1 can repress epithelial to mesenchymal transition (EMT) by inhibitory phosphorylation of transcription factor Snail, a master switch of EMT. Supported by this award, we have performed experiments on molecular, cellular, mice xenografts and transgenic levels and conclude that PKD1 is a context-dependent tumorigenesis and metastasis repressor or enhance. Specifically, PKD1 is a metastasis repressor in luminal type breast cancer and loss of PKD1 in luminal type cells converts them into basal-like cells; and PKD1 is an enhancer of tumorigenesis in basal-like breast cancer. Based on current available data, conditional knockout PKD1 in mouse mammary tissue does not disrupt mammary development and does not induce tumorigenesis.

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Introduction:

The aim of this project is to better understand the mechanisms that regulate breast cancer cell plasticity and origins of breast cancer heterogeneity. Breast cancer is a highly diverse group of cancers and consists of at least 5 different subgroups. Furthermore, patients with Intra-tumor heterogeneity due to the presence of cancer cells with variable phenotypes such as different degrees of basal-like and luminal features increase the complexity of treatment. The cellular origins of intra-tumor heterogeneity have been the subject of many recent studies. Both the cancer stem cell hypothesis and the clonal evolution model have been proposed to describe the establishment and maintenance of intra-tumor heterogeneity. In my own work, I have collected some evidence to support an alternative view, i.e. cancer cells have plasticity that manipulating certain signaling pathways can cause inter-conversion among different types of cancer cells. In other words, intra-tumor heterogeneity can arise from a subset cancer cells upon microenvironmental conditions and/or cell-intrinsic signaling. Our focus is the function of protein kinase D1 (PKD1), which is a serine/threonine kinase. Supported by this award, we have performed experiments on molecular, cellular, mice xenografts and transgenic levels to illustrate that (1) a classical breast cancer luminal cell line can be switched into basal cell-like by rerouting cellular signaling pathways; (2) PKD1 is a gatekeeper for breast cancer luminal cell identity. Loss of PKD1 in luminal cells results in epithelial to mesenchymal transition and leads to basal-like phenotypes in breast cancer.

Body:

Aim (1): Protein kinase D1 (PKD1) is necessary for maintenance mammary luminal cell identity and loss of PKD1 results in reprogram luminal cell fate.

I. PKD1 IS A METASTASIS SUPPRESSOR GENE IN LUMINAL MCF7 CELL.

To understand the role of PKD1 in breast cancer epithelial cells, we established MCF7 stable cell line that constantly express Snail-wild type (WT), Snail-S11E and –S11V. Snail, a well-known transcription repressor of the key epithelial adherens junction protein E-cadherin and an EMT inducer [1, 2], is a phosphorylation substrate of PKD1 on residue serine-11 (S11) [3]. The MCF7 is a typical luminal cell line with $CD24^{hi}CD44^{lo}$ phenotype (**Fig 1A**). The resulting cell lines MCF7/Snail-WT and Snail-S11E (mimics constant phosphorylation) did not display a significant switch of $CD24^{hi}CD44^{lo}$ phenotype (**Fig 1A**). In contrast, the Snail-S11V (mimic non-phosphorylation on S11) cells were predominantly $CD44^{hi}/CD24^{lo}$, suggesting PKD1 phosphorylation determines Snail transcription activity. Next, we knocked down PKD1 expression in MCF7 cells using two individual shRNAs and showed a dramatic switch to $CD44^{hi}/CD24^{lo}$ population (**Fig 1B**).

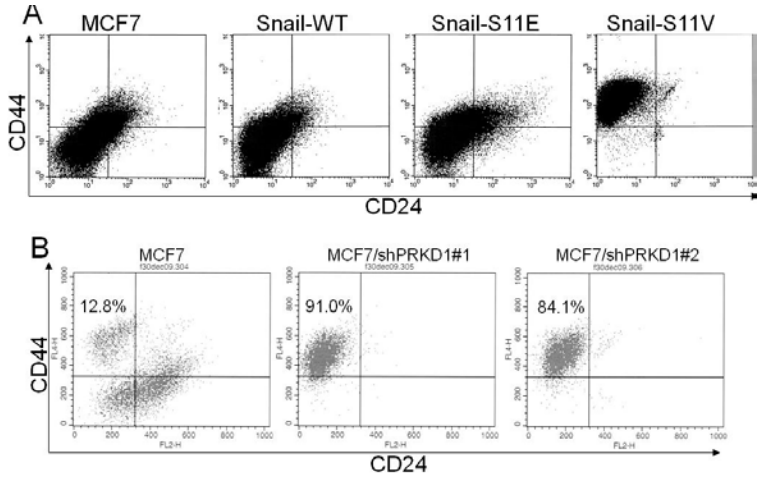
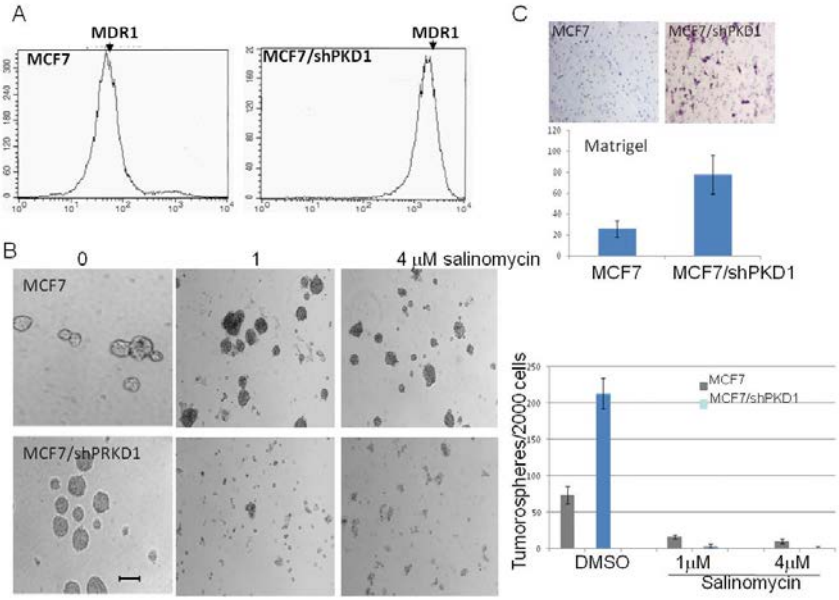


Fig 1. Flow cytometry analysis of breast cancer stem cell surface markers. *(A) MCF7 stable cell lines that express wild-type (WT), S11E (mimic serine-11 phosphorylation), S11V (non-phosphorylation) mutant of Snail, a known EMT inducer [1, 2]. PKD1 can phosphorylate Snail at serine-11 and inhibit Snail transcription activity [3]. (B) MCF7 stable cell lines that express shPKD1 (from Open Biosystems. Two individual sequences were used). These data show that majority of MCF7 cells were transformed from luminal type cells ($CD24^{hi}CD44^{lo}$) into $CD24^{lo}CD44^{hi}$ basal-like cells when loss of PKD1 or expression of Snail S11V.*

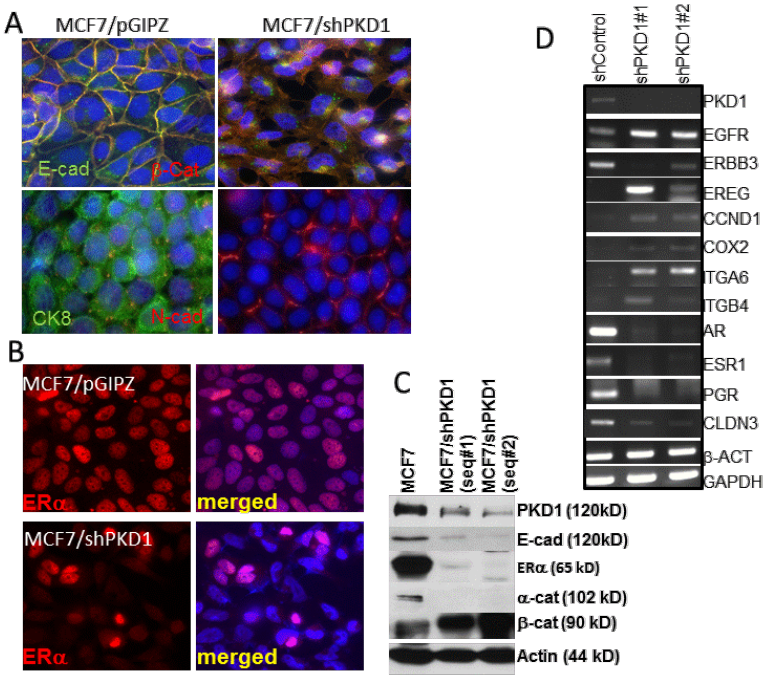
Since cancer stem cells (CSC) are within the $CD24^{lo}CD44^{hi}$ population [4], we tested stem cell activity of the MCF7/shPKD1 cells. As shown in **Fig 2**, knockdown PKD1 in MCF7 cells led to elevated expression of CSC marker MDR1 (ABCB1), to gain invasion ability on Matrigel assay and to form tumorspheres more efficiently (about 3 times more) than did the parental epithelial cells. Salinomycin, an antibiotic that preferentially inhibits CSC proliferation [5], efficiently reduces the formation of tumorspheres, indicating the MCF7/shPKD1 cells are enriched for stem cell activity.

Fig 2. Inhibition of PKD1 generates CSC properties in MCF7 cells*. (A) Flow cytometry analysis of MDR1, a stem cell biomarker. (B) Tumorsphere formation and salinomycin sensitivity. (left) Representative images of tumorspheres from MCF7 or MCF7/shPKD1 cells in the presence of 0, 1 or 4 μM of salinomycin. The bar equals to 50 μm.* (right) *Quantification of tumorsphere formation. Bars denote the standard deviation for n=3.* (*C) Matrigel assay to test cancer cell invasion.*



CSCs are generated in some cases are one of the products of an EMT, indicating that these cells possess a more mesenchymal phenotype [6, 7]. We compared the molecular phenotypes of MCF7 and MCF7/shPKD1. We found that knockdown PKD1 induced elevated levels of mesenchymal biomarkers (N-cad and β-catenin) as well as loss the key epithelial biomarkers E-cadherin and CK8, and altering β-catenin distribution (**Fig 3A**). Massive parallel DNA sequencing of basal-like breast cancer revealed that α-catenin gene (CTNNA1) was deleted in all metastatic samples [8], indicating α-catenin plays a vital role in metastasis of basal-like cancers. α-catenin recently was identified as a tumor suppressor in basal-like breast cancer by inhibiting NF-κB signaling [9]. Our data indicates that knockdown PKD1 results in reduced expression of α-catenin (**Fig 3C**), suggesting potential clinical aggressiveness of MCF7/shPKD1 cells. More importantly, the MCF7/shPKD1 cells show reduced expression of estrogen receptor (ERα/ESR1, **Fig 3B**-D). Clinically, the triple negative (ERα, PgR and Her2) breast cancer overlaps (not identical) with molecularly defined basal-like cancer. Since MCF7 cell does not express Her2, the lack of expression of ESR1 and PgR (Fig 3D and Table 1) literally make the MCF7/shPKD1 cell a triple-negative cancer cell.

Fig 3. Loss of PKD1 induces EMT and down-regulation of ERα. *(A and B) Immunofluorescence imaging of E-cadherin, N-cadherin, Cytokerin 8 and β-catenin (A), and ERα (B) in control MCF7/pGIPZ and MCF7/shPKD1 cells. (C) Western blotting analysis of indicated proteins in MCF7 and MCF7/shPKD1 cells (two different shRNA targeting PKD1 were used). (D) RT-PCR analysis of gene expression in MCF7 and MCF7/shPKD1 cells. Gene symbol: ERα, estrogen receptor α; PgR, progesterone receptor; AR, androgen receptor; NR3C1, glucocorticoid receptor; EGFR, epidermal growth factor receptor, ERBB3, epidermal growth factor receptor 3; EREG, epiregulin (a member of EGF family); ITGA6; Integrin alpha 6; ITGB4, Integrin beta 4; Cox2, Cyclooxygenase 2; IL 1B, Interleukin 1B; CCND1; cyclin D1; CLDN3, Claudin 3.*



Using microarray gene expression analyses to search the changes of expression in MCF7/shPKD1 cells, we found there are total 733 genes whose expression has a two-fold change. **Table 1** is a selective list of characteristic genes whose

expression was altered by knockdown of PKD. Among down-regulated genes, there are ERα and its responsive genes, epithelial differentiation genes, pro-apoptosis and DNA repair genes. Many of the up-regulated genes have been known for association with malignancy, poor prognosis and overall survival, such as PLAT, PLAU, CAV1/2, ITGA6, ITGB4 and some are basal cell signature genes. Validation of the microarray data for some genes by RT-PCR is in **Fig 3D**.

Gene symbol	Name	si1/MCF7	si2/MCF7 (log 2 ratio)	
PRKD1	PKD1	-3.24	-2.49	
ESR1	estrogen receptor 1	-13.69	-10.63	ER-regulated genes
PGR	progesterone receptor	-3.92	-3.79	
TFF1	trefoil factor 1	-12.63	-12.44	
IGFBP4	insulin-like growth factor binding protein 4	-4.4	-4.14	
PRLR	prolactin receptor	-5.27	-5.75	
BMP7	bone morphogenetic protein 7	-5.2	-4.96	
RERG	RAS-like, estrogen-regulated, growth inhibitor	-6.07	-6.61	
MUC1	mucin 1	-7.8	-7.84	
GATA3	GATA binding protein 3	-18.89	-14.9	epithelial differentiation
XBP1	X-box binding protein 1	-2.42	-1.77	
ELF5	E74-like factor 5 (ets domain transcription factor)	-10.33	-9.31	
SPDEF	SAM pointed domain containing ets transcription factor	-7.07	-6.14	
PDCD4	programmed cell death 4 (neoplastic transformation inhib	-4.99	-4.21	
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	-20.72	-21.34	pro-apoptosis
TP53INP1	tumor protein p53 inducible nuclear protein 1	-3.24	-3.11	
AR	androgen receptor	-2.46	-2.46	
CTNNA1	catenin, alpha	-3.63	-4.29	
BRIP1	BRCA1 interacting protein C-terminal helicase 1	-3.02	-3.78	DNA repair
RAD51C	RAD51 homolog C (S. cerevisiae)	-2.3	-1.93	
CLDN3	Claudin 3	-2.22	-1.62	
PLAT	plasminogen activator, tissue	2.6	1.32	basal cell signature genes associated aggressive tumor features
PLAU	plasminogen activator, urokinase	2.6	1.84	
CAV1	caveolin 1	3.27	1.41	
CAV2	caveolin 2	2.46	1.5	
TM4SF1	transmembrane 4 L six family member 1	2.51	1.79	
EREG	epiregulin	23.6	11.3	
LAMB3	laminin, beta 3	2.69	2.25	
LGR5	leucine-rich repeat-containing G protein-coupled recepto	2.55	2.14	
TGFBR3	transforming growth factor, beta receptor III	2.08	1.89	
TGFB2	transforming growth factor, beta 2	1.52	2.07	
CTGF	connective tissue growth factor	1.93	2.47	
ETV4	ets variant gene 4 (ets-related molecule)	2.27	1.89	
ETV5	ets variant gene 5 (ets-related molecule)	2.37	1.72	
ITGA3	integrin, alpha 3	2.13	1.33	
ITGA6	integrin, alpha 6	2.27	1.54	
ITGB4	integrin, beta4	2.21	1.17	
BIRC3	baculoviral IAP repeat-containing 3	2.22	1.68	
KITLG	KIT ligand, stem cell factor	2.13	1.95	

Table 1. Selected genes that up- or down-regulated by PKD1 in MCF7 cells. *Two individual shRNA targeting PKD1 were stably transfected into MCF7 cells. Genome-wide cDNA microarray (Affymetrix GeneChip Human Gene 1.0 ST Array) was performed to compare expression in MCF7 and MCF7/shPKD1. The genes in blue are down-regulated in MCF7/shPKD1. The genes in red are up-regulated in MCF7/shPKD1. The values are given as Log2 ratios.*

The change in the ERα expression with PKD1 knockdown warns a possible failure of hormone therapy in clinical, since hormone therapy is one of the most effective treatments for most ERα positive patients, but triple negative breast cancer patients are insensitive to this therapy. To test this possibility, we compared growth MCF7 and MCF7/shPKD1 cells in the presence of estrogen 17β-estradiol. The parental MCF7 cell is response to E2 stimulation at low concentration of estrogen. Maximum stimulation reaches around 10 nM, the physiological range of human estrogen. Higher concentration of E2 generates inhibitory effects on cell growth. In contrast, the growth of MCF7/shPKD1 cell is not sensitive to E2 stimulation (**Fig 4**). The result suggests that knockdown PKD1 can lead to resistance of tumor cells to endocrine therapy.

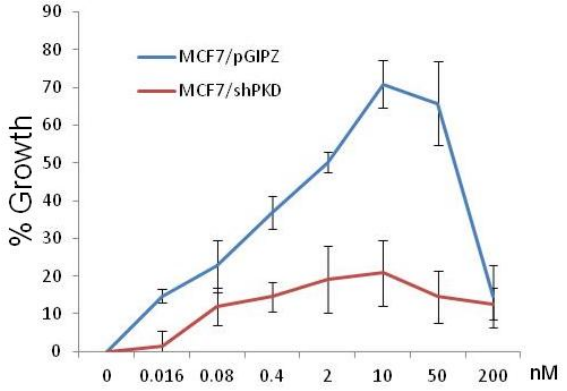


Fig 4. MCF7/shPKD1 cells lost response to estrogen stimulation. *Cells were plated in 96-well at a density of 6000-8000 cells/well, in 1% charcoal-pretreated FBS, phenol-red-free DMEM medium with indicated concentrations of E2 (17β-estradiol). The culture lasted for 5 days with media change every 48 hr with fresh test compounds. Cell density was measure with Aqueous One kit (Promega). Results were from average of triplicate samples with standard errors.*

We assessed the tumorigenicity of control MCF7/pGIPZ or MCF7/shPKD1 cells by injecting subcutaneously into female athymic nude mice. When inoculated at $5x10^6$cells/mouse, the average tumor size in MCF7/shPKD1 is significantly larger than the one in control. When inoculated at $5x10^5$ cells/mouse, the average tumor size has no difference (**Fig 5A**). In both inoculation concentrations, the tumor incidences have no significant difference.

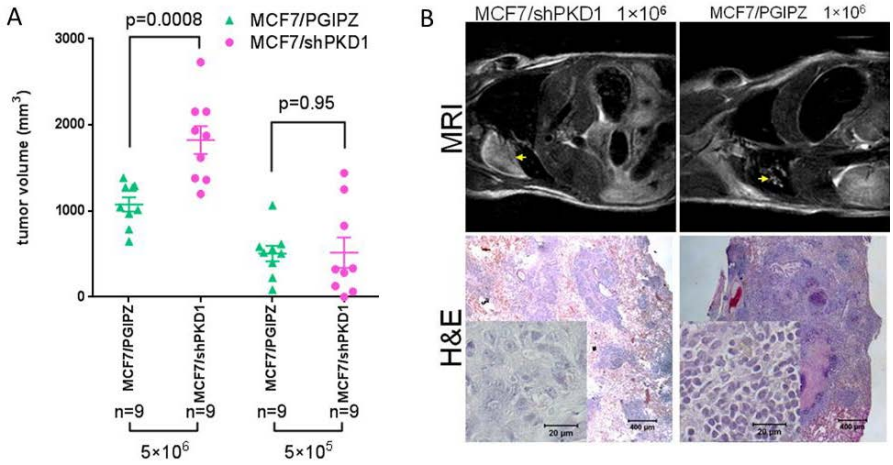


***Fig 5. Tumorigenicity of MCF7/shPKD1 cells**. (A) Tumor size and incidence in subcutaneous xenograft. Indicated numbers of cells were suspended in 1:2 diluted Matrigel:DMEM in total volume of 0.1 ml were injected into 6-8 weeks old female athymic nude mice. Tumor sizes were measured 53 days after inoculation. Numbers are mice used. (B) Xenografted mice (tain vein injection) were examined for lung tumors by using MRI imaging (top panels). Yellow arrows indicate lung tumors. (bottom) H &E staining of lung tumors. Insets are enlarged images of tumors. More IHC staining with various antibodies is in progression. All animal work was approved by UMASSMED IACUC protocol #2270.*

We also tested metastasis potentials of the MCF7/shPKD1 cell by inoculating both control and tester cells via tail vein route. Tumors were detected by MRI imaging. As shown in **Table 2**, $1x10^4$ of MCF7/shPKD1 can form lung metastatic tumor, whereas $1x10^6$ of MCF7/pGIPZ cells were needed. The pathological staining of tumor samples was sown in **Fig 5B**. From these data, we conclude that knockdown PKD1 in MCF7 cell generates more metastasis and more aggressive phenotype.

	# of cells	Inoculation	n mice	Lung Matastatic Colonization (2 month)	Tumor Incedence
MCF7/PGIPZ	1×10^6	IV	9	1	11.10%
	5×10^5	IV	9	none	0%
	5×10^4	IV	9	none	0%
MCF7/shPKD1	1×10^6	IV	9	3	33.30%
	5×10^5	IV	9	1	11.10%
	5×10^4	IV	9	1	11.10%

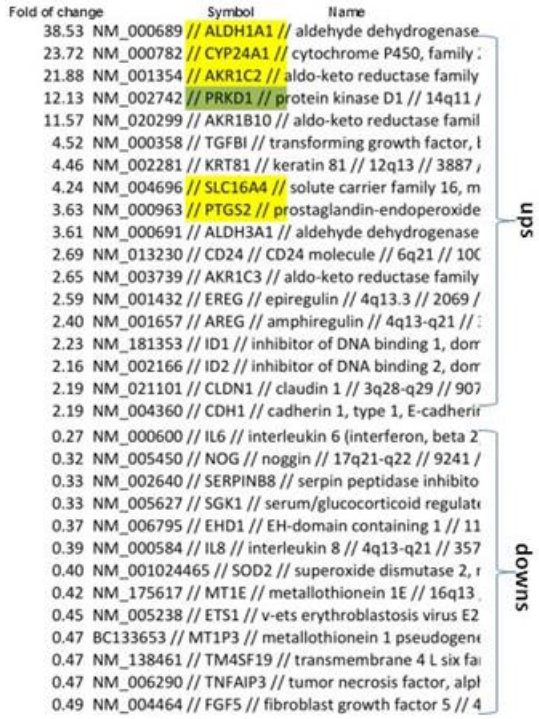
***Table 2. The MCF7/shPKD1 cells have more metastasis potential in mice xenografts**. Tail vein injection of MCF7/pGIPZ (control) or MCF7/shPKD1 cells into female athymic nude mice (6-8 weeks of age). Lung metastasis was monitored with MRI at 8 weeks after injection.*

Our data strongly suggest that PKD1 is required to maintain epithelial identity of MCF7 cell. Knockdown PKD1 results in loss of expression of ERα and Progesterone Receptor and switches MCF7 cells into basal-like cells via epithelial to mesenchymal transition (EMT). The resulting MCF7/shPKD1 cells have basal cell properties, such as molecular signature and cellular behavior. More importantly, the resulting MCF7/shPKD1 cells have more potential to form lung tumors. Our results suggest that PKD1 is a metastasis suppressor gene in luminal MCF7 cell.

II.PKD1 IS A POTENTIAL ONCOGENE IN BASAL-LIKE BREAST CARCINOMAS.

We initially hypothesized that ectopic expression of PKD1 in aggressive breast cancer basal cell line MDA-MB-231, a PKD1 negative, basal-type cell line could lower its tumorigenesis and metastasis abilities. We carried out a serial molecular, cellular and animal experiments to test the hypothesis. PKD1 was stably expressed in MB231 cells. A microarray gene expression analysis was performed. Among genes with two-fold change in expression induced by overexpression of PKD1, there are 97 genes that are up-regulated and 316 genes that are down-regulated. a selected list of genes of interest with 2-fold changes in expression is in **Table 3**. There are several types of gene of interest on the list, including stem cell specific markers (ALDH1A1 and ALDH3A1), epithelial markers (CD24, CDH1, CLDN1), EGF family members (AREG and EREG), steroid

metabolism enzymes (AKR1C2, AKR1C3 and AKR1B10), vitamin D3 metabolism (Cyp24A1), inflammation modulator PTGS2/Cox2 and lactic acid transporter SLC16A4. Of the down-regulated genes, IL6 and IL8 are inflammatory cytokines that promote cancer progression and metastasis, SGK1 has a role to inhibit apoptosis of breast cancer cells. Of particular interest, expression of androgen receptor (AR) is up-regulated by PKD1. Recently, a study suggested that the basal type breast cancer be further subtyped into five groups, one is luminal androgen receptor positive (LAR) group [10]. One characterization of the LAR group is expression of AR and response to anti-androgen therapy. In the future, we should test if the MB231/PKD1 response to androgen stimulation.



Fold of change	Symbol	Name
38.53	NM_000689 // ALDH1A1 //	aldehyde dehydrogenase
23.72	NM_000782 // CYP24A1 //	cytochrome P450, family
21.88	NM_001354 // AKR1C2 //	aldo-keto reductase family
12.13	NM_002742 // PRKD1 //	protein kinase D1 // 14q11 /
11.57	NM_020299 // AKR1B10 //	aldo-keto reductase famil
4.52	NM_000358 // TGFBI //	transforming growth factor,
4.46	NM_002281 // KRT81 //	keratin 81 // 12q13 // 3887 /
4.24	NM_004696 // SLC16A4 //	solute carrier family 16, m
3.63	NM_000963 // PTGS2 //	prostaglandin-endoperoxide
3.61	NM_000691 // ALDH3A1 //	aldehyde dehydrogenase
2.69	NM_013230 // CD24 //	CD24 molecule // 6q21 // 10(
2.65	NM_003739 // AKR1C3 //	aldo-keto reductase family
2.59	NM_001432 // EREG //	epiregulin // 4q13.3 // 2069 /
2.40	NM_001657 // AREG //	amphiregulin // 4q13-q21 //
2.23	NM_181353 // ID1 //	inhibitor of DNA binding 1, don
2.16	NM_002166 // ID2 //	inhibitor of DNA binding 2, don
2.19	NM_021101 // CLDN1 //	claudin 1 // 3q28-q29 // 907
2.19	NM_004360 // CDH1 //	cadherin 1, type 1, E-cadherin
0.27	NM_000600 // IL6 //	interleukin 6 (interferon, beta 2
0.32	NM_005450 // NOG //	noggin // 17q21-q22 // 9241 /
0.33	NM_002640 // SERPINB8 //	serpin peptidase inhibito
0.33	NM_005627 // SGK1 //	serum/glucocorticoid regulat
0.37	NM_006795 // EHD1 //	EH-domain containing 1 // 11
0.39	NM_000584 // IL8 //	interleukin 8 // 4q13-q21 // 357
0.40	NM_001024465 // SOD2 //	superoxide dismutase 2,
0.42	NM_175617 // MT1E //	metallothionein 1E // 16q13
0.45	NM_005238 // ETS1 //	v-ets erythroblastosis virus E2
0.47	BC133653 // MT1P3 //	metallothionein 1 pseudogen
0.47	NM_138461 // TM4SF19 //	transmembrane 4 L six fa
0.47	NM_006290 // TNFAIP3 //	tumor necrosis factor, alpl
0.49	NM_004464 // FGF5 //	fibroblast growth factor 5 // 4

Table 3. List of top genes that up- or down-regulated by overexpression of PKD1 in MB231 cells

The expression profiling data was collected from microarray analyses using Affymetrix GeneChip (Human Gene 1.0 ST Array). PKD1 (green) is overexpressed by stable transfection. Genes highlighted by yellow color have been validated by RT-PCR in Fig 6.

Fig 6. Validation of gene expression in MB231/GFP and MB231/PKD1. *RT-PCR was performed to confirm the up-regulated genes by overexpression include two cancer stem cell markers (ALDH1A1 and ALDH1A3). Other interesting top up-regulated genes include Cyp24A1, a vitamin D3 metabolism enzyme; SLC16A4, a lactic acid transporter; AKR1C2, a progesterone metabolism enzyme; AKT1 and PI3KCA are oncogenes to promote cell survival; Cox2 is involved in inflammation. The expressions of ERα and ERβ are not affected.*

Nevertheless, the resulting MB231/PKD1 displays many cellular behaviors that are different from parental MB231 cell, for example, growth rate and resistance to anti-cancer drugs. The MB231 cell line is a basal type cell line and is resistant to EGFR inhibitor erlotinib, mitotic inhibitor paclitaxel and DNA damaging agent cisplatin. However, over-expression of PKD1 makes the cell line sensitive to erlotinib and cisplatin (**Fig 7**). The triple negative breast cancer patients have fewer choices of therapeutic agents.

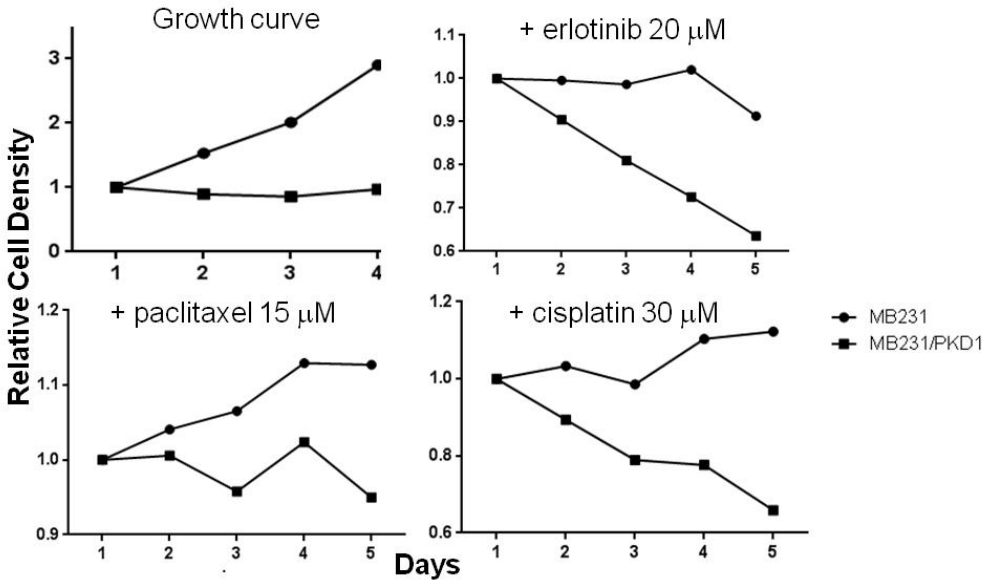


Fig 7. MB231 and MB231/PKD1 have differential sensitivity to therapeutic agents. *Cells were plated into 96-well plates at 2000-4000 cells/well. After overnight culture, indicated agents were added and continued for 5 days. Cell viability was measured by MTS method. Each point represents the average of three samples.*

In addition, the MB231/PKD1 cells display higher effectiveness on survival and proliferation on clonogenic assays in vitro (**Fig 8**), suggesting the MB231/PKD1 cell has more tumorigenicity potential in vivo. Indeed, MB231/PKD1 cells formed tumors with an increased frequency and were a substantially bigger size relative to MB231 controls when xenografted subcutaneously into female nude mice (**Fig 9**). Xenograft generated by tail vein injection also indicated that MB231/PKD1 has more metastatic potential (**Table 4**). These data suggest that PKD1 has a role in promoting tumor formation and metastasis in MB231 cell by a mechanism that reverse the MB231 cell back to stem cell like state with expression of AR. More molecular and cellular experiments are needed to confirm. Thus, we have modified our hypothesis that PKD1 is a context-dependent tumorigenesis and metastasis repressor or enhance. The dual role of a gene acting as tumor promoter and tumor suppressor also exist in literature, for example, cell polarity protein Par3 has such a dual role, depending on the tumor type involved in skin tumorigenesis [11].

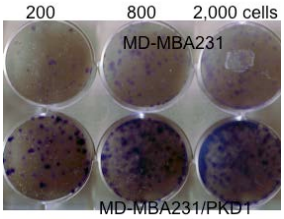


***Fig 8. Clonogenic survival assays**. MB231 and MB231/PKD1 cells were plated in a 6-well plate at 200, 800 and 2000 cell/well and allowed to grow for 12 days. The cellular colonies were stained with Quike Diff (Fisher Scientific). The MB231/PKD1 cell has more colony formation ability.*

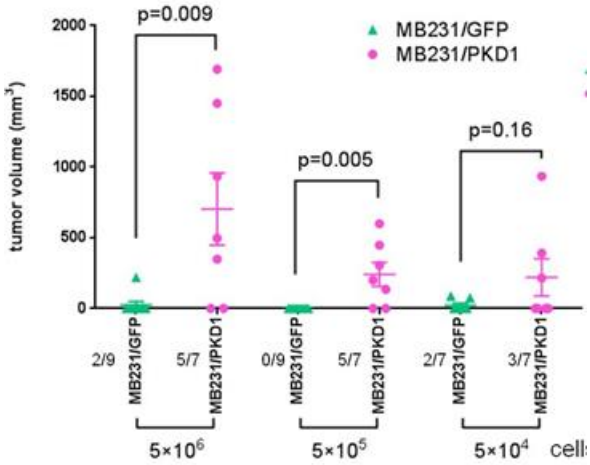


***Fig 9. Tumor incidence and size for MB231 and MB231/PKD1 in mouse subcutaneous xenografts**. The indicated numbers of cell were injected in 6-8 weeks old, female athymic Nude mice via subcutaneous route. Tumor sizes were measured at the 53rd day (9 weeks) after injection. Initial results indicate that MB231/PKD1 has more tumor formation potential than MB231. More data analysis and IHC study will be performed.*

***Table 4. Table 2. The MB231/PKD1 cells have more metastasis potential in mice xenografts**. Tail vein injection of MB231 (control) or MB231/PKD1 cells into female athymic nude mice (6-8 weeks of age). Lung metastasis was monitored with MRI at 8 weeks after injection.*

	# of cells	Inoculation	n mice	Lung Matastatic Colonization (2 month)	Tumor Incedence	Number of foci / single mice
MB231/GFP	1×10^6	i.v.	9	2	22.20%	1 – 4
	1×10^5	i.v.	9	1	11.10%	2
	1×10^4	i.v.	9	none	0%	none
MB231/PKD1	1×10^6	i.v.	9	4	44.40%	2 – 12
	1×10^5	i.v.	9	2	22.20%	1 – 2
	1×10^4	i.v.	9	1	11.10%	1

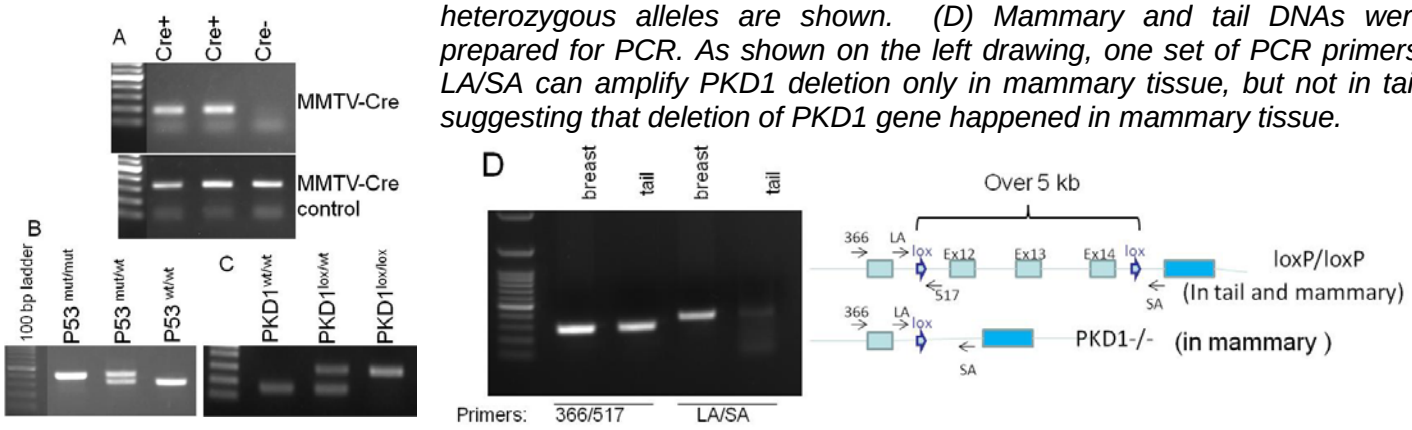
III. A POSSIBLE MOLECULAR MECHANISM FOR THE CELL-CONTEXT DEPENDENT PKD1 ACTIVITY.

To explain the dual role of PKD1 in MCF7 and MB231 cells, we think that one of our PKD1 studies provide some useful clues. In brief, PKD1 binds to E-cadherin in epithelial cell membrane and facilitate PKD1 activation. The kinetics of PKD1 activation is different in the presence or absence of E-cadherin. More importantly, PKD1 has different substrate phosphorylation profiles in the presence or absence of E-cadherin and it may suggest that PKD1 has distinct function in different cell types. A manuscript of the finding has been submitted to peer-reviewed journals for publication and is in Appendix 1.

Aim (2): PKD1 function in mouse mammary tissue development. We will generate mammary tissue specific PKD1 deletion mice and examine abnormality in mammary gland.

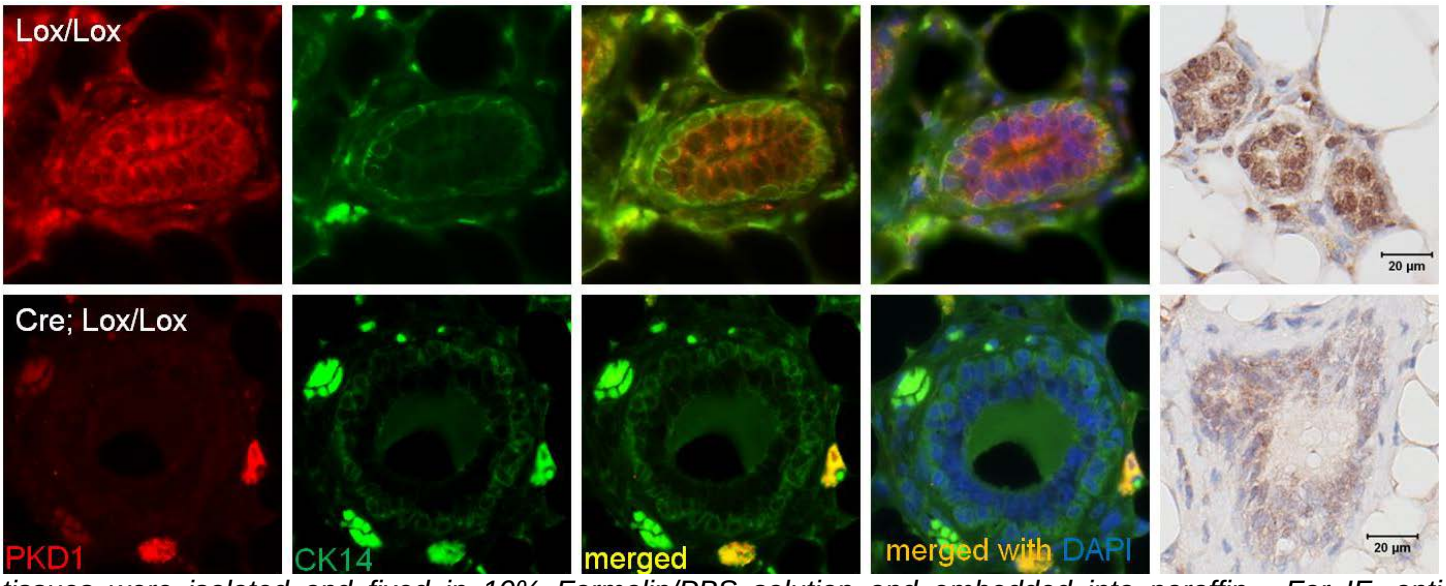
(a) Generation of *PKD1* knockout mice and *PKD1/p53* double knockout mice. We have bred male MMTV-Cre mouse (NCI mouse repository, #01XA9, line F) with female PKD1 Lox/Lox [12] (kindly provided by Dr. Eric N. Olsen, University of Texas Southwestern Medical Center). Further breeding of heterozygous Cre+; PKD1 Lox/+ mice leads to generate Cre+; PKD1 Lox/Lox mice, which have PKD1 deletion in Cre+ tissues including mammary glands. The *p53* mutant flox mouse was purchased from Jackson Lab (#008462) was mated with Cre+; PKD1 lox/+ to generate Cre+; PKD1$^{lox/+;}$ p53 $^{mut/wt}$ mice, who were further mated among siblings to generate PKD1/p53 double knockout mice.
We have established genotyping PCR protocols for Cre, PKD1 Lox/Lox and P53 mut/mut (**Fig 10**) and we are able to breed mice with expected genotypes.

Fig 10. PCR based genotyping. *DNA extracted from mouse tail was used to perform genotyping to identify Cre (A), P53 mutant (B) and PKD1 Lox (C) alleles. In detecting P53 and PKD1 alleles, homozygous and heterozygous alleles are shown. (D) Mammary and tail DNAs were prepared for PCR. As shown on the left drawing, one set of PCR primers, LA/SA can amplify PKD1 deletion only in mammary tissue, but not in tail, suggesting that deletion of PKD1 gene happened in mammary tissue.*

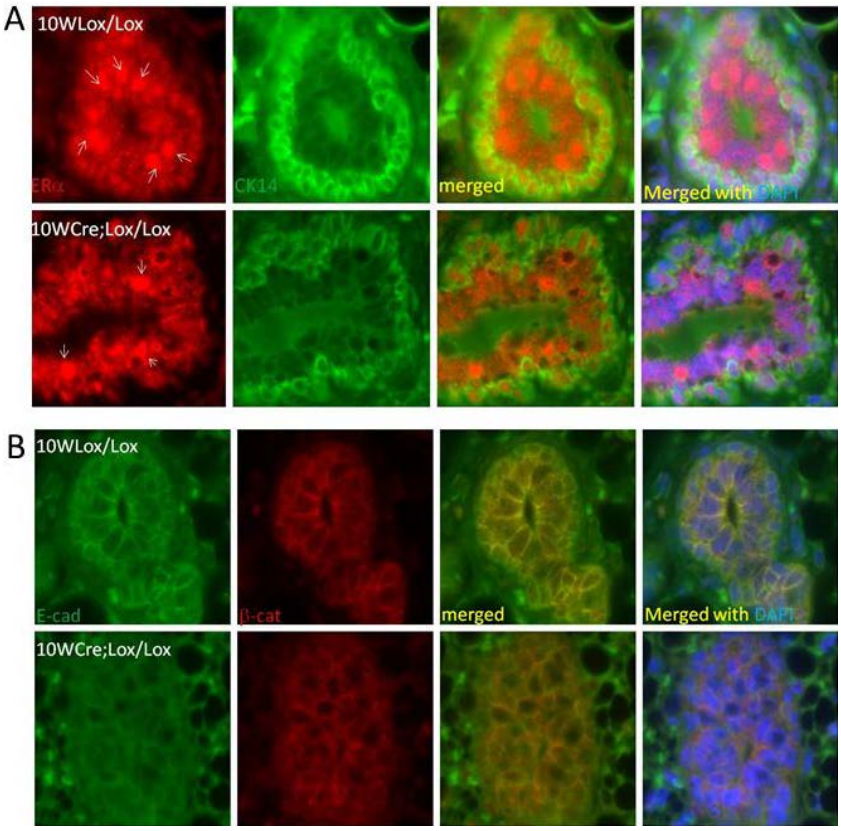


(b) Collection of mammary tissues at different stages and examination. After genotyping, female mice with desired genotypes were allowed to grow to certain age. We have collected PKD1 knockout mice with ages from 3 to 33 weeks. Currently, we have 17 PKD1 KO (Cre+; PKD1 Lox/Lox) mice, 11 littermate control (PKD1 Lox/Lox) and 4 Cre+ littermate mice. Immuno-fluorescence staining was performed to confirm PKD1 deletion from mammary tissue (**Fig 11**). PKD1 mainly expresses in luminal area, but also weakly expresses in basal area (overlapping with CK14 expression zone).

Fig 11. PKD1 protein locates in luminal cells only in a mouse mammary. *Ten weeks old mouse mammary tissues were isolated and fixed in 10% Formalin/PBS solution and embedded into paraffin. For IF, anti PKD1antibody A20 (Santa Cruz Biotech) was used at 1:400 dilution. Basal cell biomarker cytokeratin 14*



(CK14) antibody (clone LL02) was used at 1:400 dilution. For IHC, anti-PKD1 antibody (Sigma Chemicals, cat# K4892) was used at 1:200. The result suggests, for the first time in literature, that PKD1 protein expresses in luminal cells only. We have tried four (4) different PKD1 antibodies, the above two antibodies give the best results.

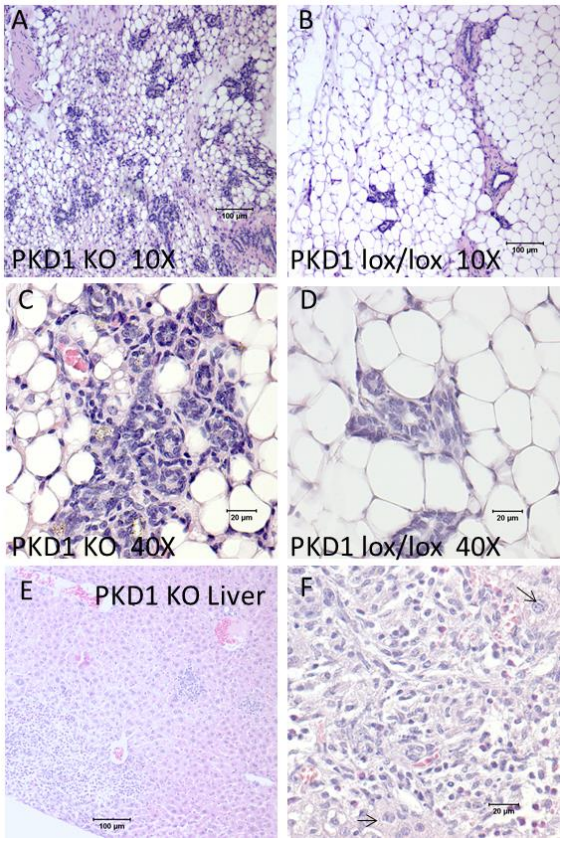


We also examined ERα (**Fig 12A**) and E-cadherin (**Fig 12B**) protein level in PKD1 knockout mice. The control mouse (10 weeks old PKD1 Lox/Lox) has more highly stained cells than those in PKD1 KO (white arrows). Expression of E-cadherin in control displays typical membrane staining pattern, but not in PKD1 KO. These data suggest that PKD1function is required to maintain ERα and E-cadherin expression in luminal cells.

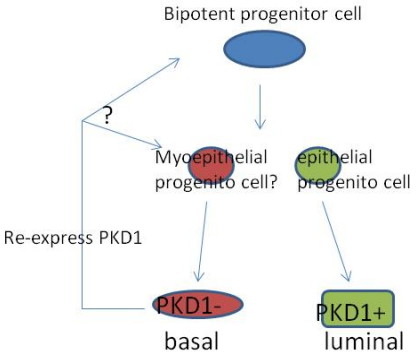
Fig 12. Expression of ERα and E-cadherin proteins are decreased in mammary gland of PKD1 KO mice*. (A) ERα antibody (MC20, Santa Cruz Biotech) was used at 1:200 dilution for IF. The E-cadherin antibody (E36, from BD) and β-catenin antibody (H108, Santa Cruz) were used at 1:200 dilution. Quantitative measurement and statistical analysis method are needed to develop.*

Two of the 17 PKD1 knockout mice developed breast and liver cancers and died at ages of 16 and 17 months, respectively. In contrast, 11 littermate control (PKD1 Lox/Lox) and 4 Cre+ littermate mice are healthy. H&E staining of PKD1 KO and control samples confirm tumor presence (**Fig 13**).

Fig 13. PKD1 KO mice develop breast and liver tumors. *Mammary (A-D) and liver tissues (E and F) were stained with H & E. Mammary tissues from PKD1 knockout (A and C) displayed over-proliferation. In contrast, the control PKD1 lox/lox (B and D) has normal pattern. Tumors were also found in liver tissue from PKD1 KO. Bars represent either 100 μm (A, B and E) or 20 μm (C, D and F). The arrows in F indicate liver cells.*



CONCLUSION: We have conducted comprehensive studies at molecular, cellular and animal model levels to illustrate our hypothesis that luminal type of breast cancer cell can be converted into basal-like cell by manipulating PKD1 signaling. In normal mouse mammary development, PKD1 expression is limited in luminal cell only. PKD1 functions in maintenance of epithelial cell identity. In luminal cancer cell line MCF7, loss of PKD1 results in downregulation of E-cadherin, ERα and ERα-regulated genes. Knockdown PKD1 in MMTV-Cre mice, the luminal cells (CK8 positive) express less ERα and E-cadherin. More importantly, we have observed that 2/17 PKD1 KO mice develop breast and lung tumors, suggesting PKD1 plays a critical anti-cancer role in mammary gland. According to currently available data, we propose a mode of PKD1 action (left). The function of PKD1 in breast cancers is dual, in luminal type cancer, it has a cancer suppressor role; in basal-like cancer, it promotes tumor. Effects for further analysis of normal/cancer stem cells are hampered by lack of single distinct stem cell markers with anatomical position information, such as LGR5 in small intestine stem cell [13]. We have noted that many triple negative cancers and many basal-like cancer cell lines express PKD1, it is interesting to know how PKD1 contribute to tumor progression and metastasis. For future work on PKD1, I suggest: (1) test other promoter driven Cre (such as WAP-cre) to knockout PKD1 and study PKD1 function during pregnancy and lactation; (2) knockin PKD1 into mammary basal cells using CK14-Cre mouse with P53 mutant background. This mouse model may mimic some human triple breast cancer with P53 mutant and expressing PKD1 and this approach will help to understand how PKD1 acts in basal-type cancer cells; (3) using existing small molecule inhibitors for PKD1 to treat breast cancer in mouse models. As we suggest that expression of PKD1 in basal-like cancer cells promotes tumor progression, it is possible to inhibit PKD1 will suppress tumor; and (4) data mining to surrogate correlation of PKD1 expression with clinical outcomes of breast cancer patients with different genetic background and subtype of tumors.



KEY RESEARCH ACCOMPLISHMENTS:

- Propose an alternative hypothesis, other than stem cell or clonal evolution to explain the origin of intra-tumor heterogeneity, i.e., basal-like breast cancer can arise from terminally differentiated luminal cancer cells. Supported by this grant, we have collected experimental data to support our hypothesis.
- Illustrate Protein kinase D1 role in breast cancers and may help designing personal therapeutic regimen for PKD1 positive and negative breast cancer.
- To our best knowledge, we are the first to carry out the first PKD1 knockout experiment in mouse mammary tissue.

REPORTABLE OUTCOMES:

- One manuscript has been submitted for peer-reviewed journal, another is in writing and preparing.
- One postdoctoral researcher (Dr. Zhuo Li) has been trained and now appointed as an oncological surgeon in a China hospital.
- Multiple cell lines (two for MCF7/shPKD1, one for MDA-MB231/PKD1, one for SKBR3/PKD1). Multiple mouse knockout PKD1 mammary tissues and parallel control have been collected.

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Appendix 1: Manuscript submitted for peer-reviewed publication

OXFORD UNIVERSITY PRESS Journal of Molecular Cell Biology

E-cadherin facilitates protein kinase D1 activation and subcellular localization

Journal:	*Journal of Molecular Cell Biology*
Manuscript ID:	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Li, Zhuo; China Medical University, Surgery Zhang, Chuanyou; University of Massachusetts Medical School, Surgery Chen, Li; University of Massachusetts Medical School, Surgery Li, Guosheng; Shandong Academy of Agriculture Sciences, Qu, Ling; Shandong Academy of Agriculture Sciences, Balaji, KC; Wake Forest University, Urology Du, Cheng; University of Massachusetts Medical School, Surgery
Keyword:	Protein kinase D1, E-cahderin, interaction

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E-cadherin facilitates protein kinase D1 activation and subcellular localization

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Running title: PKD1 associates with E-cadherin

Keywords: Protein kinase D1, E-cadherin, activation, protein interaction, subcellular localization, epithelial to mesenchymal transition

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ABSTRACT

Protein kinase D 1 (PKD1) is a serine/threonine kinase implicated in the regulation of diverse cellular functions including cell growth, differentiation, adhesion, and cell motility. The current model for PKD1 activation involves diacylglycerol binding to the C1 domain of PKD1 and translocation to membrane where PKD1 is phosphorylated and activated by protein kinase C (PKC). We show that the intracellular domain of membrane protein E-cadherin binds to PKD1 via the second cystein-rich (C1b) and catalytic domains. The binding leads to subcellular redistribution of PKD1 and has functional significance, as PKD1 has dramatically different kinetics and substrate phosphorylation profiles during activation in the presence or absence of E-cadherin. Furthermore, artificial targeting PKD1 to plasma membrane leads to PKD1 activation in a PKC-independent manner, indicating that membrane attachment is sufficient to activate PKD1. Knockdown of PKD1 in lung epithelial cell line A549 results in epithelial to mesenchymal transition with changes in biomarker expression and cellular behavior. These observations suggest that E-cadherin facilitates PKD1 activation and that it is required for efficient plasma membrane localization and extend our previous understanding of PKD1 regulation and E-cadherin's signaling functions.

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INTRODUCTION

When a cell responses to various external environmental stimuli, signaling pathways serve as on-off switches to transmit the message in. The molecular mechanisms that control the specificity of signaling have been studied extensively. Protein kinase C family has provided a good example (Newton, 2009; Steinberg, 2008). PKC isoforms serve diverse (and in some cases opposing) functions in cells, however, they share general mechanisms in activation and substrate phosphorylation. Classic PKC members can be activated by diacylglycerol (DAG) and calcium signals. How can PKC multiple isoforms in the cell react differently to same second messenger signal input? It is at least in part as results of protein-protein interaction leading unique subcellular localization. Several classes of PKC interacting proteins have been identified, including Receptors for activated C kinases (RACKs), Substrate-binding proteins (SBPs) and Proteins that interact with C kinase (PICKs) (Pawson and Scott, 1997). These adaptor proteins have in common the ability to bind specific PKC isozymes and connect them to a particular organelle or site in the cell (Schechtman and Mochly-Rosen, 2001).

Protein kinase D1 (PKD1), the founding member of PKD kinase family, is crucial for development since PKD1 knockout is embryonic lethal in mice (Fielitz et al., 2008) and the PKD1 function is not complemented by two close family members, PKD2 and PKD3. PKD family is classified within the calcium/calmodulin kinase (CAMK) superfamily, however, PKDs share many structural and regulatory similarities to the PKC family and previously identified as PKC family members (Rozengurt et al., 2005; Wang, 2006). The PKD family members share certain basic structural features, including cysteine-rich region (C1 region, subdivided into C1a and C1b regions), a pleckstrin homology (PH) domain and catalytic domain (Rozengurt et al., 2005; Wang, 2006). Like PKC, PKDs remain none/low catalytic activity in unstimulated cells through an autoinhibition mediated by the PH domain (Iglesias

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and Rozengurt, 1998). Previous studies leads to a general model of activation of PKD1 (Rozengurt et al., 2005). In brief, PKCs and PKD1 are recruited to the membrane surface in response to second messenger DAG. Activation of PKD is dependent on PKC activity to phosphorylate two key serine residues at PKD1 activation loop (Ser-744 and -748 in mouse PKD1, Ref (Rozengurt et al., 2005)). Activated PKD1 can autophosphorylate Ser-916 residue, an event which is often used as a surrogate marker for PKD1 activity. More recent studies show that PKD1 activation is more complicated. For example, G protein-coupled receptors induce not only a rapid PKC-dependent activation phase involving Ser-744 and -748 phosphorylation, but also a prolonged PKC-independent activation phase with Ser-748 autophosphorylation (Jacamo et al., 2008; Sinnett-Smith et al., 2009). Furthermore, dextran sulfate, a potent PKD1 agonist, increases high level S916 phosphorylation, but low/none Ser-744/748 phosphorylation (Rybin et al., 2012).

Although phosphorylation plays a major role in PKD1 signaling efficiency or specificity, docking interactions with protein scaffolds or protein substrates also may contribute to the allosteric control of PKD1 activity (Steinberg, 2011). The A-kinase anchoring protein AKAP-Lbc assembles an activation complex including PKD1, PKCeta and PKA in cardiomyocytes. In this protein complex, PKD1 is activated by nearby localized PKC and release from the complex mediated by PKA activity (Carnegie et al., 2004). PKD1 was previously reported to co-localize and co-purify with E-cadherin, a major protein component in cell-cell adherens junctions (Jaggi et al., 2005). In this study, we show E-cadherin facilitates PKD1 plasma membrane translocation and activation.

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RESULTS

E-cadherin physically interacts with PKD1 and knockdown E-cadherin decreases PKD1 activity. PKD1 was previously reported to co-localize with E-cadherin, a major protein component in epithelial cell-cell adherens junctions (Jaggi et al., 2005). Since PKD1 was also reported to bind directly to β-catenin, a known protein associated with E-cadherin (Du et al., 2009), it is uncertain if PKD1 association with E-cadherin is through a direct or indirect binding. To explore the possibility of direct interaction, we performed a yeast 2-hybrid assay using individual PKD1 domains, C1a, C1b, PH and catalytic domain with E-cadherin intracellular domain. Both C1b and catalytic domain of PKD1 can directly bind to E-cadherin intracellular domain (Fig 1A). The C1b domain of PKD1 has been known for binding to DAG and plasma membrane attachment during PKD1 activation (Rozengurt et al., 2005; Van Lint et al., 2002). Reciprocal co-immunoprecipitation experiments confirm that PKD1 and E-cadherin in C4-2/PKD1 cells are associated with each other (Fig 1B), suggesting that the two proteins are within the same complex in mammalian cells. Since PKD1 is a protein kinase and its catalytic domain binds to E-cadherin, we tested if E-cadherin cytoplasm domain can be a phosphorylation substrate for PKD1 *in vitro*. E-cadherin cytoplasm fragment fused to GST protein was cloned and expressed in *E.coli* cells and was purified by glutathione beads pull down. The purified E-cadherin cytoplasm fragment is not phosphorylated by PKD1 (data not shown). However, we found that expression a shRNA vector that targets E-cadherin in LNCaP, an E-cadherin positive prostate cancer cell line, can reduce PKD1 activation as judged by S910 autophosphorylation (Fig 1C, equivalent to mouse PKD1 residue S916). Furthermore, β-catenin threonine-120 is a phosphorylation site for PKD1 (Du et al., 2009), western blot with the phosphorylation specific β-catenin antibody (pT120) confirms that PKD1 trans-phosphorylation activity is higher in the presence of E-cadherin (Fig 1C).

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Artificial targeting PKD1 to membrane activates PKD1 in the absence of simulations. To further study how binding to E-cadherin might be required for PKD1 activation *in vivo*, we tested whether membrane recruitment of PKD1 might play a role in activation by artificially targeting it to the membrane. We made a fusion protein consisting of the first N-terminal 20 amino acid residues of neuromodulin and PKD1 (mem-PKD1, Fig 2A). This neuromodulin fragment contains a palmitoylation signal that targets fusion proteins to membranes (Liu et al., 1993). When this construct transfected into SW480 cells, an E-cadherin negative cell line the mem-PKD1 protein mainly localizes to plasma membrane and small fraction of the protein attaches to organelle membranes (Supplementary Figure S1A). Immunoblotting for pS916 shows that the mem-PKD1 is activated in the absence of stimulation (lane 5, Fig. 2B). In contrast, the endogenous PKD1 remains inactive (lane 5, Fig. 2B) until Bryostatin-1was added (lane 6).

Both C1a and C1b domains of PKD1 can bind to DAG, however, C1b domain is responsible for the majority of DAG binding (Iglesias et al., 1998). Mutation of mouse PKD1 proline-287 residue (equivalent human PKD1 residue P281), a critical DAG binding site within C1b domain abolishes DAG/phorbol ester binding, abolishes PKD translocation from cytosol to plasma membrane and prevents PKD activation (Iglesias et al., 1998; Jacamo et al., 2008; Rey et al., 2004). Indeed, the P281G mutant PKD1 (Fig 2A) is not able to be activated by Bryostatin 1 as indicated by phosphorylation status of S916 (Lane 4, Fig 2D). DAG has three functions in activating PKD1: (1) promoting PKD1 membrane translocation; (2) anchoring PKD1 on membrane via hydrophobic tails of DAG; and (3) activating PKCs as upstream kinase activator. We generated P281G mutant with the artificial membrane attachment from neuromodulin (P281G/mem-PKD1, Fig 2A) and examined its activation. Like mem-PKD1, the P281G/mem-PKD1 becomes activated in the absence of stimulation

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(lane 7, Fig 2D), suggesting targeting wild type or P281G mutant PKD1 to membrane mimics the DAG-induced membrane translocation, activating responses normally induced by DAG.

Previous studies suggest that PKCs as upstream kinases to activate PKD1 in canonical pathway (Rozengurt et al., 2005). Since the mem-PKD1 can be autophosphorylated without exogenous stimulation and upstream PKCs activation, we hypothesized that the activation of mem-PKD1 in resting cells is a PKC-independent event. Incubation with PKC inhibitor GF1 can prevent activation of endogenous PKD1 (lane 6, Fig 2E), but does not block activation of the mem-PKD1 in the absence or presence of Bryostatin 1 (Lanes 5 and 6, Fig 2E), suggesting that the auto-activation of mem-PKD1 is PKC-independent. Like wild-type PKD1, the mem-PKD1 still requires Bryostatin 1 stimulation to translate to other subcellular compartments (compare Fig 2B and 2C for distribution).

E-cadherin binding affects PKD1 subcellular localization. The wild type GFP-tagged PKD1 (GFP-PKD1) is predominately localized in cytoplasm of E-cadherin negative C4-2 cells (Fig 3B and 3D). Co-transfected with full-length E-cadherin, the two proteins are co-localized on cytoplasm and membrane (Fig 3C). When activated by Bryostatin 1, PKD1 and E-cadherin are still co-localized (Fig 3C). We also used enriched subcellular fractionations to examine PKD1 subcellular localization. In resting C4-2 cells, PKD1 is totally a cytosol protein and a small portion of PKD1 redistribute to membranes and nuclei upon stimulation (Fig 3D). In the presence of E-cadherin, a minor fraction of PKD1 is on membrane in the C4-2/E-cad resting cells and dramatic amount of PKD1 redistribute to membranes and nuclei upon stimulation (Fig 3D).

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To further examine how PKD1 subcellular localization is affected by E-cadherin, we tested two additional E-cadherin constructs and one PKD1 construct. The E-cadherin cytoplasm fragment CTF2 (Fig 3A) is a partially proteolytic cleavage product that naturally occurs during oncogenesis and apoptosis (van Roy and Berx, 2008). Overexpression of CTF2 results in co-localization with wild type PKD1 in cytoplasm in the absence or presence of Bryostatin (Fig 4A). The IL2R-EcadC (Fig 3A) is a chimera that fuses the extracellular and transmembrane domains of the Interleukin-2 Receptor α subunit to the cytoplasm domain of E-cadherin (Gottardi et al., 2001). The IL2R-EcadC and wild type PKD1 proteins co-localize in resting cells and re-distribute to paranuclear upon stimulation (Fig 4B). We also made a PKD1 construct that expresses nuclear localized PKD1 by inserting SV40 nuclear localization signal (NLS) at the N-terminus of PKD1 (Fig 2A). This NLS-PKD1 localizes in nuclei in resting cells and re-distributes to cytoplasm upon stimulation (Fig 4C and Fig 2D). In the presence of CTF2 (Fig 4D) or IL2R-EcadC (Fig 4E), NLS-PKD1 is partially associates with CTF2 and IL2R-EcadC in resting cells, but it becomes overwhelmingly co-localized with the two E-cad variants after stimulation. From these data, we summarize that (1) no matter where the initial locations (cytoplasm, nuclear or plasma membrane) of PKD1 in resting cells, PKD1 become relocated upon stimulation (Figs 2C and 2D); and (2) in the presence of E-cadherin or its variants, activated PKD1 relocation always associates with E-cadherin or its variants (Fig 3 and 4).

E-cadherin binding changes the kinetics of PKD1 activation and substrate phosphorylation profiles. We compared the time course of PKD1 activation in C4-2 and C4-2/E-cad cells. When treated with Bryostatin 1, the activation course of PKD1 is dramatically different in the presence and absence of E-cadherin. PKD1 in the control C4-2 cells was slowly activated and it took 2 hours to reach maximum activation as judged by pS916 (lanes 1-10, Fig 5A). In contrast, PKD1 in the C4-2/E-cad cells responded

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quickly and reached peak activation in about 5-10 minutes (lanes 11-20, Fig 5A). Like PKC isoforms, prolonged stimulation (> 4 hours) results in PKD1 protein degradation (Leontieva and Black, 2004). These data suggest that PKD1, in the absence of E-cadherin, has a slow response to stimulation and maintains maximum activation for a short time period. In contrast, E-cadherin binding confers PKD1 a rapid activation and sustainable maximal activity (Fig 5B).

Next, we ask if the binding of active PKD1 to E-cadherin on plasma membrane will change its substrate phosphorylation profile due to subcellular conpartmentation. The antibody pMOTIF recognizes preferred PKD1 phosphorylation motif LxRxxpS/T (where p represents phosphorylated residues) (Doppler et al., 2005). The time courses for PKD1 substrate phosphorylation of C4-2/GFP and C4-2/Ecad cells recognized by pMOTIF antibody are quite different (Fig 5B). First, the high molecular weight substrates (100-200 kDa) in C4-2/GFP cells have none phosphorylation at resting and become gradually phosphorylated over time. Meanwhile, the same proteins in C4-2/Ecad cells show basal phosphorylation at resting, and maximal phosphorylation can last for at least 4 hours. Second, a protein band around 35 kDa (arrowhead in Fig 5C) is maximally phosphorylated between 30 minutes to 4 hours and is one of the major bands in the C4-2/GFP cells, but it is only weakly phosphorylated in C4-2/Ecad cells. Third, the PKD1 phosphorylates unique substrates in C4-2/GFP cells but not in C4-2/Ecad cells (indicated by * in Fig 5C). Finally, two bands show strong phosphorylation in resting C4-2/Ecad cells (arrows in Fig 5C). Activation of PKD1 inhibits the phosphorylation of the two bands as short as 5 minutes. Although the exact mechanism is unknown, one explanation is that the PKD1 basal activity is responsible for phosphorylation in resting cells; the translocation of PKD1 upon stimulation disassociates PKD1 from its substrates and therefore restricts substrate availability.

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Loss of PKD1 induces epithelial to mesenchymal transition (EMT), increased cell migration and drug resistance. To study the biological significance of this E-cadherin-PKD1 interaction on cellular behaviors, we knocked down PKD1 expression using shRNA in lung cancer cell line A549, an E-cadherin positive cell line. The protein level of epithelial biomarker E-cadherin decreases dramatically with loss of E-cadherin (Fig 6A), the expression of mesenchymal biomarkers N-cadherin, vimentin and α-smooth muscle actin increases (Fig 6B), suggesting PKD1 function is critical for maintenance of epithelial identity. Knockdown PKD1 increases A549 cell migration in wound healing assay (Fig 6C).

The Epidermal Growth Factor Receptor (EGFR) kinase inhibitors, including gefitinib and erlotinib, show potent therapeutic efficacy in non–small cell lung cancers. However, acquired drug resistance is a major clinical problem to use these inhibitors. Recent studies show that EMT plays a role in acquired resistance to gefitinib or erlotinib in lung cancer (Ahmad et al., 2013; Chung et al., 2011; Suda et al., 2011; Yauch et al., 2005). The A549 cell line is moderately sensitive to erlotinib (Yauch et al., 2005). Knockdown of PKD1 in A549 cell is associated elevated resistance to erlotinib (Fig 6D). The EMT enhanced drug resistance has also found in chemotherapy drug docetaxel (Fig 6E).

DISCUSSION

In this report, we found PKD1 protein has different behaviors in the presence or absence of membranous protein E-cadherin. We showed that the two proteins physically bind to each other and E-cadherin is required for PKD1 optimal membrane attachment and activation. We emphasize the role of E-cadherin in regulation of PKD1 activity and localization. Our major findings are: (1) PKD1 has distinct kinetics of activation in the presence or absence of E-cadherin (Fig 5A and 5B). This difference in kinetics may physiologically cause significant difference for PKD1 in response to short life signals in a cell; (2) E-

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cadherin affects PKD1 subcellular localization (Fig 3 and 4). In addition, our data shows that the soluble E-cadherin cytoplasm fragment CTF2 can also bind to and alter PKD1 subcellular localization (Fig 4A). Although the biological significance of this alteration is still to be evaluated, it is tempting to assume that PKD1 may play different functions in the presence of E-cadherin CTF during tumorigenesis; and (3) the PKD1 substrate phosphorylation shows dramatically distinct patterns with or without E-cadherin (Fig 5C), suggesting that PKD1 may phosphorylate different substrates and regulate different signal pathways and biological events in context of E-cadherin. Identifying of unique PKD1 substrates can be a future pursue.

Our study also suggests that membrane anchored PKD1, unlike cytoplasmic soluble PKD1, maintains a constant activated status (Fig 2B-2C) and does not require DAG and PKCs for activation. This is a novel finding and has not been described the current model of PKD1 activation (Rozengurt et al., 2005). This kind of signal cascade, however, has many similar examples in literature. Yeast pheromone response pathway, for example, involves the activation of MAP kinase cascade through Gβγ complex and scaffold protein Ste5. Pheromone exposure promotes membrane translocation of Ste5, along with associated inactive MAP kinases, and binds to membrane Gβγ and thus the MAP kinases get accessed to proximate upstream kinase Ste20. Membrane recruitment of Ste5 by Gβγ complex can be bypassed with artificial targeting Ste5 to the plasma membrane in the absence of pheromone (Pryciak and Huntress, 1998).

In non-epithelial cells, PKD1 may partner with other proteins to achieve optimal activation. One example is B cell antigen receptor (BCR) engagement which leads to activates both PKD1 and Bruton's tyrosine kinase (Btk) (Sidorenko et al., 1996). It has been reported that PKD1 physically associates with

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Btk (Johannes et al., 1999) and Btk-deficiency diminishes PKD1 activation (Sidorenko et al., 1996), suggesting Btk is required for PKD1 activation. However, a naturally occurred dominant negative form of Btk (*xid*, which contains point mutant R28C) does not abrogate PKD1 activation in B cells (Haxhinasto and Bishop, 2004) and the authors proposed that the role of Btk is not to directly activate PKD1, but rather to cooperate with PKD1-mediated signals as an adaptor protein. The PKD1 protein contains multiple regulatory domains which can recruit interacting proteins. For example, the C-termini of PKD1 and PKD2 have a PDZ-binding motif and both isoforms can bind to membrane localized PDZ domain-containing protein NHERF-1 (Kunkel et al., 2009). Importantly, agonist-evoked activation of PKD in the presence of the NHERF-1 scaffold is rapid and sustained compared with cytosolic PKD. In HEK293 cells, the C1b domain of PKD1 directly binds to G-protein Galphaq subunit, and the binding contributes to long-lasting activation of PKD1 on plasma membrane (Oancea et al., 2003). Therefore, our results provide an alternative explanation for the diverse functions of PKD1, i.e. protein-protein interactions are a mechanism for PKD1 regulation, poising PKD1 near upstream kinases and lipid regulators and near specific substrates.

Since PKD1 can suppress the function of transcription factor Snail, a known repressor of E-cadherin expression and up-regulate E-cadherin expression (Du et al., 2010), the PKD1-E-cadherin proteins form a positive feedback loop that involves transcriptional as well as kinase regulation, favoring maintenance of epithelial cell identity. Loss of PKD1 results in down regulation of E-cadheirn expression; and loss of E-cadherin expression or cancer-related proteolysis of E-cadherin that generates soluble CTF2 can alter PKD1 localization and function. Our results demonstrate that spatial and temporal organization of the PKD1 signal transduction is essential in determining the speed and precision by which signaling events occur.

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MATERIALS AND METHODS.

Plasmid constructs: The GFP-PKD1 was described previously (Jaggi et al., 2005). The P281G-PKD1 (equivalent to mouse PKD1 P287 (Iglesias et al., 1998; Jacamo et al., 2008)) which contains a point mutation in proline-281 into glycine residue in the C1b domain was generated by PCR-based mutation with Quikchange kit (Stratagene). The primer used (forward) 5'-GTCATCCACTCCTACACCCGGGCCACAGTGTGCCAGTACTGCAAG. The NLS-PKD1 was generated by inserting SV40 nuclear localization signal (NLS, with amino acid sequence PKKKRKV and nucleotides CCA AAA AAG AAG AGA AAG GTA) downstream of GFP. Mem-PKD1 was created by inserting the sequence encoding the first 20 amino acid residues of neuromodulin (MLCCMRRTKQVEKNDEDQKI, corresponding nucleotide sequence ATGCTGTGCTGTATGAGAAGAACCAAACAGGTTGAAAAGAATGATGAGGACC AAAAGATC) upstream of GFP-PKD1 (Fig 1A). Detail methods and primer sequences are available on request. The shRNA construct targeting E-cadherin (siE-cad) was described before (Du et al., 2010). E-cadherin CTF2-pcDNA3 construct (Ref (Ferber et al., 2008), Fig 3A) was kindly provided by Dr Y. Fujita, University College London. This construct contains the whole intracellular domain. IL2R-EcadC (Fig 3A) is a chimera that fuses the extracellular and transmembrane domains of the interleukin-2 receptor α subunit to the cytoplasmic domain of E-cadherin [Ref (Gottardi et al., 2001), kindly provided by Dr. C. Gottardi, Northwestern University]. For yeast two-hybrid assay, the pGBK-T7 plasmid (Clontech) containing C1a, C1b, PH and catalytic domains of PKD1 were described previously (Rao et al., 2003). E-cadC-pGAD-T7 vector contains part of trans-membrane and whole intracellular domain of E-cadherin (amino acid residues 716-883). This fragment was cloned by PCR and inserted into pGAD-T7 vector in frame. A full-length E-cadherin expression vector (Fig 3A) was obtained from Addgene.org (Plasmid

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#18804). ShRNAs targeting E-cadherin and PKD1 were purchased from Open Biosystems/Thermo Scientific.

Cell culture and immunofluorescence staining. Prostate LNCaP and lung cancer cell lines were purchased from ATCC and cultured in RPMI medium plus 10% unheated FBS. Prostate cancer cell line C4-2 was purchased from Uroco, Inc and cultured in DMEM plus 10% FBS. The stable C4-2 cell line that expresses PKD1-GFP (C4-2/PKD1) was described before (Jaggi et al., 2005). The Colon cancer cell line SW480 (from ATCC) was cultured in L15 medium plus 10% FBS. A C4-2 cell line that stably expresses full-length E-cadherin (C4-2/Ecad) was established by antibiotic selection of transit transfected C4-2 cells with the E-cadherin expression vector. Bryostatin 1 (from Sigma Chemicals) was added to culture media to a final concentration of 20 nM. In Fig 1C, general PKC inhibitor GF 109203X (GF1, from Sigma Chemicals) was added to cell culture 1 hour before adding Bryostatin 1 to a final concentration of 1 μM. Erlotinib and Docetaxel were purchased from LC labs. For immunofluorescence imaging, cells cultured on glass discs were fixed in 5% Formalin in PBS for 10 minutes at room temperature. After washing, primary antibody was diluted in PBS solution with 0.3% Triton X-100 and 10 mg/ml BSA and incubated with fixed cells from 1 hour to overnight following by incubation with secondary antibody conjugated with appropriate dyes (all from Jackson ImmunoResearch). Images were taken by an Olympus IX-51 microscope equipped with SPOT software. Transient transfection was performed with Lipofectamine 2000 (Invitrogen). The enrichment of cytosol, nuclear and membrane fraction was achieved by using a protein fraction enrichment kit (ITSI Biosciences).

Cell migration (wound-healing) assays. A549 and derivative cells were seeded at $5x10^5$ per well on a 6-well plate and allowed to grow for another day to a near confluence. Scratches were made using a sterile pipet tip and the wells gently washed and replaced with fresh medium for overnight culture.

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Drug sensitivity test. A549 and derivative cells were seeded at 3,000 cells in 100 μL per well on 96-well plates and cultured for a day before adding drugs. Erlotinib or Docetaxel were diluted at 2-fold in 10 μL and added to each well in triplicate. Cell density measured 3 days after adding drugs using CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (from Promega).

Electrophoresis and Western blotting. Cultured cells were lysed in RIPA buffer (Alcaraz et al., 1990) containing protease and phosphatase inhibitor cocktails (Sigma Chemicals). The protein samples were separated on 10% SDS-PAGE and transferred to PVDF membranes using a semi-dry transfer devise (Thermo Scientific) at constant current model (0.2 A). The membrane was then incubated with TBST (50 mM Tris-HCl, pH7.4, 100 mM NaCl and 0.2% Tween-20) plus 3% nonfat dry milk to block non-specific binding for 1 hour at room temperature. Incubation with primary antibodies was performed at 4 C for overnight. An E-cadherin antibody recognizes the intracellular domain (clone C36) was purchased from BD Transduction Laboratories and an antibody that recognizes the extracellular domain was from Santa Cruz Biotechnology. PKD1 and phosphorylated serine-916 (pS916) PKD1 antibodies were purchased from Epitomics/Abcam. β-catenin, N-cadherin, Vimentin, α-Smooth muscle actin, β-actin, α-tubulin and GFP antibodies were purchased from Santa Cruz and Epitomics/Abcam. The development and characterization of β-catenin phosphorylated Threonine-120 antibody was previously described (Du et al., 2012). A phospho-(Ser/Thr) PKD1 substrate antibody which preferably detects LXR(Q/K/E/M)(M/L/K/E/Q/A)S*XXXX (Doppler et al., 2005) was purchased from Cell Signaling. The densitometric quantification of immunoblotting was carried out with Image J software.

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Figure Legends

Figure 1. E-cadherin interacts with PKD1. (A) Yeast 2-hybrid assay for direct interaction of PKD1 and E-cadherin. Individual PKD1 domain indicated in the figure and E-cadherin intracellular domain were used for testing. Plate on right side is grown on non-selective medium and the plate on the left is grown on selective medium. Schematic of PKD1 structure is shown at lower panel. (B) Co-precipitation of PKD1 and E-cadherin. The C4-2/PKD1 cells stably overexpress GFP tagged wild type PKD1 and induce E-cadherin expression (Du et al., 2010). C4-2/PKD1 cell lysate is used for reciprocal IP. (C) Knockdown E-cadherin reduces PKD1 activation. ShRNA targeting E-cadherin or control (siLuc) were transit transfected into E-cadherin positive cell line LNCaP. Cells were cultured for another 48 hours and treated with 20 nM of Bryostatin 1 for 30 minutes. Cell lysates were used for Western blotting analysis for indicated antibodies.

Figure 2. Artificial targeting to plasma membrane activates PKD1. (A). Schematic of PKD1 constructs used in this study. Wild-type PKD1: full length human PKD1 tagged with GFP at the N-terminus. Mem-PKD1: inserting the first 20 amino acid residues of neuromodulin that contains a palmitoylation signal and leads the fusion proteins to membranes upstream of GFP-PKD1. P281G-PKD1: PKD1 containing a point mutation on proline-281 to glycine. P281G/mem-PKD1: mem-PKD1 containing a point mutation of P281G. NLS-PKD1: PKD1 with a nuclear localization signal. (B and C) Subcellular localization of PKD1 constructs in SW480 cells. GFP-tagged wild type (left), mem-PKD1 (middle) and NLS-PKD1 (right) were transit transfected into SW480 cells.

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Representative immunefluorescent images are taken for resting cells (B) and Bryostatin 1 (20 nM) treated cells (D). PKD1 protein is in green and nucleus is in red. (D) Targeting of PKD1 to the membrane activates PKD1 without stimulation. Indicated constructs were transiently transfected into C4-2 cells for 48 hours. Bryostatin 1 was added at final concentration of 20 nM for 30 minutes. (Top panel) Immunoblotting with PKD1 pS916 antibody. Endo- and exo- represent endogenous and GFP-tagged exogenous PKD1, respectively. The endogenous PKD1 can be used as a wild type PKD1 control. (Lower panel) immunoblotting for total PKD1. Data comes from a single experiment, with similar results obtained in another experiment. (E) Activation of membrane attached PKD1 is PKC-independent. C4-2 cells were transfected as in (D). The transfected cells were pre-treated with general PKC inhibitor GF1 for 2 hours (final concentration 2.5 μM) before Bryostatin was added. (Top panel) Immunoblotting with PKD1 pS916 antibody. (Lower panel) immunoblotting for total PKD1. Data comes from a single experiment.

Figure 3. E-cadherin affects PKD1 subcellular localization. (A) Schematic of E-cadherin constructs used in this study. (top) wild type E-cadherin; (middle) E-cadherin soluble cytoplasm fragment CTF2; and (bottom) the CTF2 fused to the transmembrane domain of Interleukin 2 receptor. (B and C) PKD1 associates with E-cadherin. GFP tagged wild type PKD1 was either transit transfected alone (B), or co-transfected with full-length E-cadherin (C) and cultured for another 48 hours. Bryostatin (20 nM final concentration) was added for 30 minutes before fixation. Representative immunofluorescence images are shown. Nucleus is in blue. (D) Western blot of PKD1 in C4-2 and C4-2/E-cadherin cells. Subcellular fractionation enrichment of cytosol (C),

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nuclear (N) and total membrane (M) were prepared from resting and Bryostatin 1 stimulated cells (final concentration of 20 nM for 30 minutes). E-cadherin, a-tubulin and Lamin A/C are fraction markers and/or loading control.

Figure 4. E-cadherin associates with PKD1. E-cadherin soluble fragment CTF2 and chimera IL2R-EcadC were co-transfected with either wild-type PKD1 (A and B) or a nuclear-localized PKD1 (D and E). Cells were treated with or without Bryostatin 1. PKD1 protein is in green and nucleus is in blue.

Figure 5. E-cadherin confers rapid and sustained activation for PKD1 and unique substrate phosphorylation profiles. C4-2 cells that stably express GFP or full-length E-cadherin were treated with 20 nM Bryostatin 1 for indicated times. Cell lysates were used for immunoblotting for total and active (pS916) PKD1 (A), PKD1 phosphorylated substrate (C). All results are from a single experiment, with similar results obtained in another experiment. (B) PKD1 activation was quantified by Image J software. The chart was based single experiment.

Figure 6. Loss of PKD1 induces epithelial to mesenchymal transition (EMT), and alters cell behavior. Lung epithelial cell line A549 were transfected with two individual shRNA constructs targeting PKD1. Stable expression cells were selected by antibiotic resistance. (A) Western blotting of indicated proteins. (B) Immunofluorescent images of E-cadherin and N-cadherin expression. (C) Wound healing assays for A549 and A549/shPKD1. (D) Cell viability on treatment with Erlotinib. (E) Cell viability on treatment with Docetaxel.

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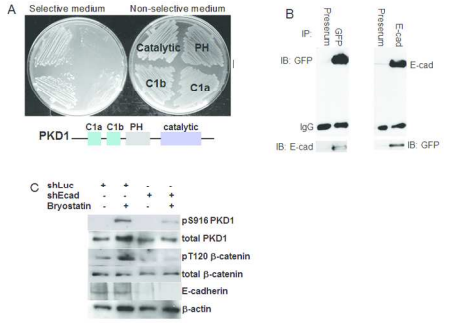
Fig 1



E-cadherin interacts with PKD1.
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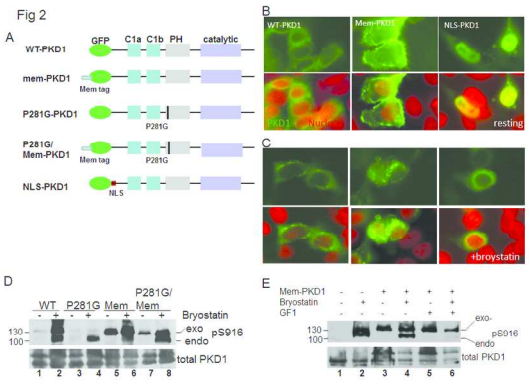

Artificially targeting to plasma membrane activates PKD1.
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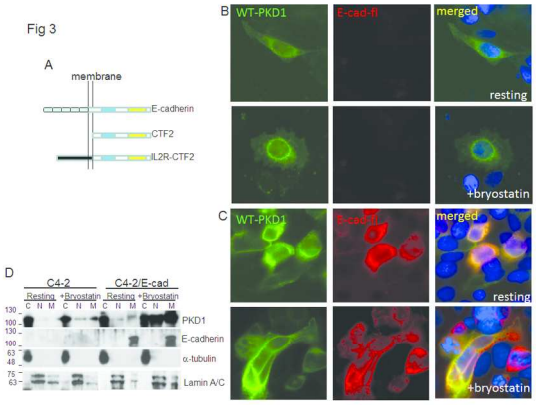

E-cadherin affects PKD1 subcellular localization
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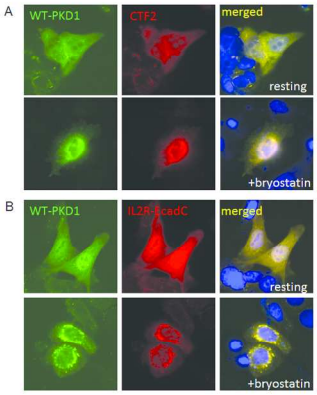
Fig 4



. E-cadherin associates with PKD1
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Fig 4 (continued)

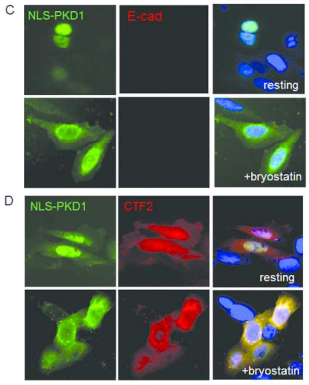

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Fig 4 (continued)

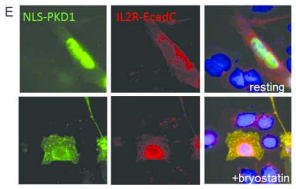

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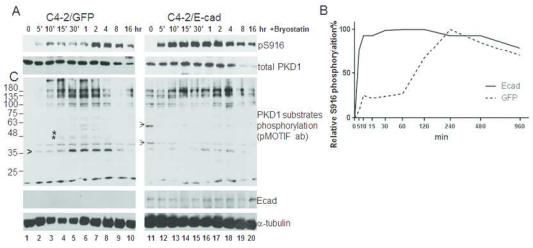
Fig 5



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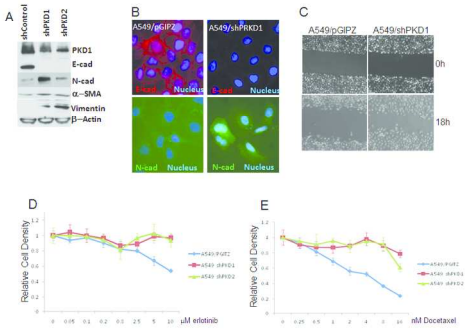

Loss of PKD1 induces epithelial to mesenchymal transition (EMT), and alters cell behavior
152x101mm (300 x 300 DPI)

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